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- (51) Int. Cl<sup>6</sup>  
C12N 15/12, 5/10, 1/21, C12P 21/02, C07K 14/50, A61K 37/36
- (11) International Publication No.: WO97/20929  
(43) International Publication Date: June 12, 1997
- (21) International Application No.: PCT/JP96/03579  
(22) International Application Date: December 6, 1996
- (30) Priority Data:  
Japanese Patent Application No.: Hei 7/345689, December 7, 1995 JP  
Japanese Patent Application No.: Hei 8/103240, March 28, 1996 JP  
Japanese Patent Application No.: Hei 8/214378, July 24, 1996 JP
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- (81) Specified Countries:  
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Published Documents Attached: International Search Report

## FOR INFORMATION

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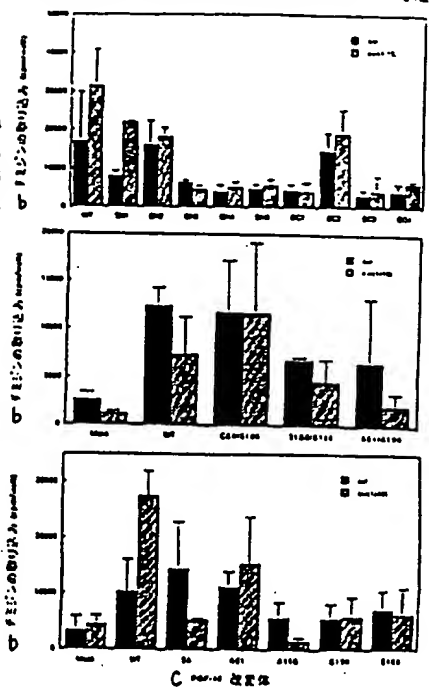
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(54) Title: Fibroblast Growth Factor FGF-10

(57) Abstract

A recombinant FGF-10 obtained by introducing an expression vector into which a DNA encoding a specific amino acid sequence has been integrated into a host cell, incubating the transformant thus obtained, and allowing it to produce the aimed protein. This recombinant FGF-10 is applicable to drugs and reagents utilizing the cell growth-promoting effect thereof.

A RECOMBINANT FGF-10 OBTAINED BY INTRODUCING AN EXPRESSION VECTOR INTO WHICH A DNA ENCODING A SPECIFIC AMINO ACID SEQUENCE HAS BEEN INTEGRATED INTO A HOST CELL, INCUBATING THE TRANSFORMANT THUS OBTAINED, AND ALLOWING IT TO PRODUCE THE AIMED PROTEIN. THIS RECOMBINANT FGF-10 IS APPLICABLE TO DRUGS AND REAGENTS UTILIZING THE CELL GROWTH-PROMOTING EFFECT THEREOF.



a ... Mitogenic activity of FGF-10 modification on FRSK cells  
b ... Thymidine uptake (cpm/well)  
c ... FGF-10 modification

## **Detailed Description**

### **Fibroblast Growth Factor FGF-10**

#### **Field in Industry**

The present invention relates to a novel fibroblast growth factor (in the following, abbreviated as FGF) and a method for producing the same by recombination. Furthermore, it also relates to the medical applications of the factor.

#### **Prior Art**

FGF was discovered in the 1970's as an angiogenic factor. Acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) have been studied and their structures and wide-ranging cell growth-promoting actions have been elucidated [D. Gospodarowics et al.: Nature Vol. 249, page 123 (1974); Burgess, W. H. and Maciag, T.: Annu. Rev. Biochem. Vol 58, pages 575-606 (1989); Suzuki, F.: Clinical Calcium, Vol. 4, pages 1516-1517 (1994)]. Currently, there are a total of 9 FGF species. They all have been cloned and their structures are known [Cell, Vol. 27, No. 9, pages 341-344 (1995)]. Existence of additional FGF species has been suggested.

On the other hand, based on their wide-ranging cell growth-promoting actions, aFGF and bFGF have been evaluated for their possible applications as promising therapeutic agents for the treatment of metabolic diseases of nervous, cardiovascular and bone systems. However, the usefulness in the clinic so far has not been established. The same evaluation of novel FGF species is desired.

The objective of the present invention is to provide a method for industrial production of a recombinant protein of a novel FGF after identifying then analyzing the gene.

#### **Disclosure of the Invention**

The inventors actively investigated DNAs of unknown FGF species. As a result, they successfully obtained a DNA of a totally novel FGF (in the following, abbreviated as FGF-10), thereby achieving the present invention.

As shown under the following items (1)-(15), the present invention relates to the DNA encoding FGF-10, an expression vector containing the DNA, a transformant, a method for

producing a recombinant protein by using the transformant, the recombinant protein, and medical applications of the recombinant FGF-10 protein.

(1) A nucleotide sequence encoding a fibroblast growth factor containing the amino acid sequence represented by sequence number 1 or sequence number 2, or a recombinant DNA containing a nucleotide sequence complementary to the above sequence.

(2) The DNA described under (1), containing the nucleotide sequence represented by sequence number 3 or sequence number 4 or a nucleotide sequence complementary to the above sequence.

(3) An expression vector containing the DNA described under (1).

(4) A transformant obtained by introducing the expression vector described under (3) into a host cell.

(5) The transformant described under (4), in which the host cell is an animal or E. coli cell.

(6) A method for producing a recombinant fibroblast growth factor, characterized by use of the transformant described under (4).

(7) A recombinant fibroblast growth factor containing the amino acid sequence represented by sequence number 1 or sequence number 2 or its main portions.

(8) The recombinant fibroblast growth factor containing the amino acid sequence represented by sequence number 1 or sequence number 2 or its main portions, characterized by that it is produced by the transformant described under (5) and has cell growth-promoting activity.

(9) A pharmaceutical preparation containing the recombinant fibroblast growth factor described under (7) or (8) as active ingredient.

(10) The pharmaceutical preparation described under (9) that is a therapeutic agent for the treatment of diseases of bone/cartilage or injuries of bone/cartilage.

(11) The pharmaceutical preparation described under (9) that is a therapeutic agent for wound healing.

(12) Use of the recombinant fibroblast growth factor described under (7) or (8) for the production of the therapeutic agent for diseases of bone/cartilage or injuries of bone/cartilage.

(13) Use of the recombinant fibroblast growth factor described under (7) or (8) for the production of the therapeutic agent for wound healing.

(14) A method for treating diseases of bone/cartilage or injuries of bone/cartilage by administering an effective dose of the recombinant fibroblast growth factor described under (7) or (8) to animals or humans.

(15) A method for healing wounds by administering an effective dose of the recombinant fibroblast growth factor described under (7) or (8) to animals or humans.

#### **Brief Legends to the Figures**

Figure 1 shows two primers used for the cloning of FGF-10, which are common to FGF-3, FGF-7 and FGF-10: (A) Tyr-Leu-Ala-Met-Asn-Lys; (B) Tyr-Asn-Thr-Tyr-Ala-Ser.

Figure 2 shows primers used for the isolation of FGF-10 cDNA by the Rapid Amplification of cDNA Ends (RACE) method.

Figure 3 shows a summary of the construction of plasmids from the plasmid pFGF-10 to the plasmid pCDM8-F10SP and finally to the plasmid pCDM8-F10HX.

Figure 4 shows primers and PCR conditions used to convert the sequence upstream of the translation start site to the Kozak consensus sequence.

Figure 5 shows the expression of the FGF-10 mRNA in articular tissue detected by in situ hybridization: (A) micrograph of an articular cartilage specimen; (B) micrograph of an apophysiary cartilage specimen.

Figure 6 shows a graph of the incorporation of tritium-labeled thymidine into FRSK cells. Bq, Sp and Hx of the horizontal axis represent control and supernatant samples of FGF-10-expressing COS cell culture, respectively, while the vertical axis represents the cell-associated radioactivity.

Figure 7 shows a processed image of X-ray photography of tibial cartilage from a FGF-10-treated animal in a test example.

Figure 8 shows a processed image of X-ray photography of tibial cartilage from a control animal in the test example.

Figure 9 shows a summary of the construction of a FGF-10 mutant-expressing plasmids and the preparation of fragments of the mutant DNAs.

Figure 10 shows a graph of the biological activities of the COS cell-expressed human FGF-10 and the mutants: the incorporation of tritium-labeled thymidine into FRSK cells. Concerning the horizontal axis, WT represents the wild type human FGF-10, while DN1-5, DC1-4, C84/S106, S150/S106, A51/A196, SA, A51, A196, S150 and S106 [Avr represents original culture supernatant, and Avr (1/10) represents 1/10 diluted culture supernatant] all represent FGF-10 mutants. The vertical axis represents the radioactivity associated with FRSK cells.

### **Best Form of the Invention**

In the present Detailed Description, the definition of the technical terms is as follows.

"FGF-10" is a mammalian fibroblast growth factor containing the amino acid sequence represented by sequence number 1 or sequence number 2 or the major portions. The major portions include the amino acid sequence of mature protein after the signal pre-sequence or pro-sequence is deleted from the above sequence. In other words, it is the 179 amino acid residue sequence from glutamine (Gln)-37 to serine (Ser)-215 in sequence number



1 or the 171 amino acid residue sequence from glutamine (Gln)-38 to serine (Ser)-208 in sequence number 2.

By currently known techniques, proteins with some amino acids in the sequence represented by sequence number 1 or sequence number 2 or in the major portions deleted, substituted or added can be produced, and based on knowledge obtained from random screening or studies on mutation of other members of the FGF family, mutant proteins with identical biological activity to that of FGF-10 of the present invention can be produced. As long as the mutant proteins have the fibroblast growth factor activity, they should be covered by the present invention. FGF-10 is a protein with two sites for N-glycosylation: Asn-Ser-Ser (50-52) and Asn-Thr-Ser (203-205) in the amino acid sequence represented by sequence number 1 or Asn-Ser-Ser (51-53) and Asn-Thr-Ser (196-198) in the amino acid sequence represented by sequence number 1. In general, the biological activity does not depend on glycosylation. Glycosylation can be modified or removed by the selection of host cells. Proteins with modified glycosylation should be covered by the present invention as long as they have the fibroblast growth factor activity.

The following FGF-10 mutants from (1) to (5) have identical biological activity to that of the wild type FGF-10 and hence should be covered by the present invention.

(1) FGF-10 mutants with the Cys residues of the wild type FGF-10 substituted by other amino acids (Ser or Ala), in which intramolecular or intermolecular disulfide bond does not exist.

(2) FGF-10 mutants with a Cys residue in the region conserved cross FGF family, which are generated by substituting an amino acid (Ser-84 in FGF-10) in the position conserved in other FGF species by Cys and substituting Cys-106, that is not conserved, by some other amino acid (Ser or Ala), due to the fact that one of the 2 Cys residues in the mature FGF-10 protein (Cys-106) is at a different position from the Cys residue conserved in other FGF species.

(3) Non-glycosylated FGF-10 mutants obtained by substituting the glycosylation sites (Asn-X-Y: X represents any amino acids other than Cys and Pro, while Y represents Ser or Thr) with other amino acids.

(4) FGF-10 mutants with a few to 100 amino acids deleted from the N- or C-terminus.

(5) FGF-10 mutants with amino acid sequences that can be cleaved by proteases substituted by other amino acid sequences.

More specifically, the following FGF-10 mutants from (1) to (19) are shown as examples. These mutants, as described in the practical examples, can be produced by currently known genetic engineering techniques.

(1) FGF-10 mutants with 6 out of the 7 Cys residues in the leader region (Cys-9, -19, -20, -21, -22 and -23) substituted by Ala or Ser.

(2) FGF-10 mutants with Cys-106 substituted by Ala or Ser.

(3) FGF-10 mutants with Cys-150 substituted by Ala or Ser.

(4) FGF-10 mutants with both Cys-106 and Cys-150 substituted by Ala or Ser.

(5) FGF-10 mutants with Ser-84 substituted by Cys and furthermore with Cys-106 substituted by Ala or Ser.

(6) FGF-10 mutants with Cys-9, -19, -20, -21, -22, -23, -37, -106 and -150 substituted by Ala or Ser.

(7) FGF-10 mutants with Asn-51 substituted by Ala.

(8) FGF-10 mutants with Asn-196 substituted by Ala.

(9) FGF-10 mutants with both Asn-51 and Asn-196 substituted by Ala.

(10) FGF-10 mutants with the amino acid sequence from the N-terminal amino acid (Leu-40) to Thr-50 deleted from the mature protein.

(11) FGF-10 mutants with the amino acid sequence from the N-terminal amino acid (Leu-40) to Ser-62 deleted from the mature protein.

(12) FGF-10 mutants with the amino acid sequence from the N-terminal amino acid (Leu-40) to Lys-81 deleted from the mature protein.

(13) FGF-10 mutants with the amino acid sequence from the N-terminal amino acid (Leu-40) to Lys-103 deleted from the mature protein.

(14) FGF-10 mutants with the amino acid sequence from the N-terminal amino acid (Leu-40) to Lys-137 deleted from the mature protein.

(15) FGF-10 mutants with the amino acid sequence from Asn-196 to Ser 208 deleted.

(16) FGF-10 mutants with the amino acid sequence from Gly-189 to Ser 208 deleted.

(17) FGF-10 mutants with the amino acid sequence from Glu-154 to Ser 208 deleted.

(18) FGF-10 mutants with the amino acid sequence from Gly-138 to Ser 208 deleted.

(19) FGF-10 mutants combining the deletion of amino acid sequence of any of (10)-(14) and that of (15)-(18).

"Fibroblast growth factor activity" is at least one of the various biological activities of the FGF family including cell growth-promoting activity such as cell growth-stimulating activity, hemopoietic progenitor growth-stimulating activity, angiogenic activity, etc., differentiation-modulating activity such as cell differentiation-inducing activity, extracellular matrix-modifying activity, etc., nervous cell survival-maintaining activity, etc. [Clinical Biochemistry, Vol. 38, No. 11, pages 219-221 (1994 Supplement Issue)]. The cell growth-stimulating activity for epithelial cell-derived cell lines including the rat embryo-derived epithelial cell line (FRSK cells), seen with FGF-7, is also included in the activity.

"Therapeutic agent for the treatment of injuries of bone/cartilage or diseases of bone/cartilage" is a pharmaceutical preparation for the promotion of healing of physical

injuries of bone/cartilage such as accident-caused fractures, surgical removal of bone/cartilage, etc., or for the treatment of diseases with decreased osteogenesis as a major symptom. The following (1)-(6) describe its medical applications:

(1) bone deficiency-treating agent, (2) fracture-treating agent, (3) osteoporosis-treating agent, (4) chondral-tissue healing-promoting agent, (5) articular chondral tissue-healing and -treating agent, and (6) deforming-arthritis-treating agent.

"Wound healing-promoting agent" is a pharmaceutical preparation for the promotion of healing of trauma, congelation, scald, etc. caused by physical/chemical factors derived from accidents. Intractable dermatomuscular tissue disorders such as radiation damage, skin ulcer derived from diabetes, bedsores, etc. are also included as targets of the treatment-promoting agent.

In the following, the present invention is described in more detail.

#### Production of FGF-10 gene

The DNA encoding FGF-10 of the present invention can be obtained by known genetic engineering techniques. Specifically, mRNA can be isolated from animal tissues or cells, then double-stranded cDNA can be synthesized. The cDNA can be amplified by PCR using primers and the sequence can be determined. Special kits are commercially available for all these experiments. Although there is no special limitation to tissues and culture cells as a source of mRNA, particularly rat embryo at about day 14 is used preferably. Since the expression level of the mRNA is relatively higher in lung and articular tissues, lung cells and culture cells derived from bone/cartilage can also be used. Commercially available poly(A) RNA (from Clontech) from adult human lungs can also be used easily and preferably.

It can also be cloned from cDNA or genomic DNA libraries from various species by using appropriate sequences from the DNA sequence encoding FGF-10 disclosed in the present Detailed Description as DNA probes.

DNA library is prepared as follows by standard procedures.

1. Lyophilized animal tissue is treated with RNase and protease, then high molecular weight DNA is obtained by precipitation. DNA extracts are commercially available (from Clontech, etc.).

2. By partial digestion with restriction enzyme (EcoRI, etc.), DNA fragments are obtained by ethanol precipitation.

3. The DNA fragments are inserted into  $\lambda$  phage by using DNA ligase.

4. By using commercially available in vitro packaging kit, packaging is performed, thereby obtaining a DNA library.

DNA probes are selected based on highly distinct sequences from the DNA sequences encoding the FGF proteins disclosed in the present Detailed Description. They are chemically synthesized, then labeled with  $^{32}\text{P}$ , etc.

#### Production of FGF-10 protein

As the expression vector containing the FGF-10 cDNA thus obtained, plasmid or phage is selected, that can be amplified in appropriate host cells of *E. coli*, *Bacillus subtilis*, yeast, animal or insect. For example, the vector can be pBR322 or pBR325 derived from *E. coli* [Gene, Vol. 4, page 121 (1978)], pUB110 derived from *Bacillus subtilis* [Biochem. Biophys. Res. Commun. Vol. 112, page 678 (1983)], pCDM8 that is preferable for COS cells, etc. For the insertion of cDNA into plasmid, standard procedures are described in Molecular Cloning by T. Maniatis et al., Cold Spring Harbor Lab, page 239 (1982).

The host cells are transformed by the introduction of the vector. There is no special limitation to the host cells, as long as they can produce FGF-10. Typical examples include bacteria such as *E. coli*, *Bacillus subtilis* (*Bacillus bacteria*), etc., yeast such as *Saccharomyces*, *Tolula*, *Pikia*(?), etc., animal cells such as COS cells, CHO cells, NSO cells, etc. Not only cultured insect, fungus and plant cells, but also insects, mammals and plants containing the gene for the target protein are included in the hosts.

As a procaryotic cell production system, *E. coli* or *Bacillus* is generally used. In particular, *Bacillus brevis* with a reduced level of protease production is useful as an

expression and secretion system [see Japanese Patent Application Publication Kokai No. Hei 6-296485, Japanese Patent Application Publication Kokai No. Hei 6-133782, Y. Sagiya et al.: *Applied Microbiol. Biotechnol.* Vol. 42, pages 358-363 (1994), etc.].

Desired clones are selected from the transformants by known methods such as the colony hybridization method [Gene, Vol. 10, page 63 (1980)] and DNA sequence determination method [Proc. Natl. Acad. Sci. USA, Vol. 74, page 560 (1977)]. Besides, clones can also be selected by transient expression in COS cells, followed by evaluation of the biological activity in the culture supernatants.

The biological activity of the expressed FGF-10 can be easily detected by standard methods. For example, it can be evaluated by assaying for the growth-promoting activity for epithelial cells such as the known cell line FRSK.

The plasmid containing the cloned DNA can be used directly, or after cut with restriction enzyme then inserted into an expression vector appropriate for selected host. FGF-10 protein can thus be produced in large quantities. There is no special limitation to expression method. All known techniques in this field can be used. For example, fusion expression, secretion expression or direct expression using bacteria, or expression using eukaryotic cells can be selected appropriately.

The FGF-10 protein thus produced by recombinant technology can be purified by purification techniques generally used in the biochemical field. For example, appropriate combinations of ion exchange chromatography, gel filtration, reverse phase HPLC, ammonium sulfate precipitation, ultrafiltration, SDS-PAGE, etc. can be used. For FGFs, particularly affinity chromatography using heparin, etc. as a ligand, antibody column chromatography, etc. are preferably used for large scale purification. Antibodies against FGF-10 protein, both monoclonal and polyclonal, can be produced by known techniques. Specific antibodies against FGF-10 can be used not only for antibody column, but also for immunochemical quantitative assays such as ELISA, etc.

The FGF-10 protein produced by the above methods has various biological activities including cell growth-promoting activity and thus can be used as a wound healing-promoting agent, circulation deficiency-treating agent, nervous cell survival-maintaining agent, hair

growth-promoting agent, etc. for medical applications. In particular, since its expression in chondral tissues of adult mammals, its applications as a bone disease-treating agent for the treatment of fractures, etc. and as a therapeutic agent for the treatment of injuries of chondral and connective tissues are possible. Moreover, it can also be used as a reagent for research works on cell growth promotion.

The administration of FGF-10 protein in animals and humans can be performed via normal administration routes, such as intramuscular, intravenous, subcutaneous, intraperitoneal, transdermal, etc. There is no special limitation to dose and administration frequency, depending on subject, administration route, severity of symptom, body weight, etc. In humans, normally the dose is about 1  $\mu$ g - 0.1 g per adult per day, once or more times a day. The dose form can be, for example, injection, etc. For pharmaceutical preparation, normal carriers can be used and the preparation is performed by standard procedures. Specifically, for the preparation of injection, lyophilized FGF-10 protein is dissolved in saline, then, if needed, pH adjusting agent, buffer, stabilizer, solubilizer, etc. are added thereby preparing the injection by standard procedures.

In the following, the present invention is further described in detail by way of practical examples. Nevertheless, the present invention is not to be limited to the examples.

#### Practical Example 1

##### Structural analysis of FGF-10 gene

##### Preparation of rat DNA library

mRNA was prepared by standard procedures from an entire 14 day old Wistar rat embryo [Chomczynski et al.: Anal. Biochem. Vol. 162, pages 156-159 (1987)]. The rat embryo mRNA was used as template to prepare rat embryo cDNA using random primer (6 mer) as primer and Moloney murine leukemia virus reverse transcriptase. Specifically, rat embryo poly(A) RNA (5  $\mu$ g) was incubated in a solution containing 300 units of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL), 15 units of human placenta RNase inhibitor (Wako Pure Chemicals) and 0.5  $\mu$ g of random primer (6 mer) at 37°C for 60 min, thereby obtaining the cDNA.

#### Preparation of primers common between FGF-3 and FGF-7

By comparing the amino acid sequences of known 7 human FGF species, 2 regions of amino acid sequences common between FGF-3 and FGF-7 (Tyr-Leu-Ala-Met-Asn-Lys and Tyr-Asn-Thr-Tyr-Ala-Ser) were selected, and 2 FGF primers as shown in Figure 1 were prepared.

#### Amplification of rat FGF family DNA

Rat embryo cDNA was used as template. The above 2 FGF primers and Taq DNA polymerase were used to amplify FGF family DNAs by the polymerase chain reaction (PCR) method. Specifically, a reaction solution (25  $\mu$ L) containing an appropriate amount of cDNA, 0.05 unit/ $\mu$ L of Taq DNA polymerase (Wako Pure Chemicals) and 5 pmol/ $\mu$ L of the above sense or antisense primer was subjected to 30 cycles of PCR. After the reaction, the solution was applied to 8% polyacrylamide gel electrophoresis, and the fraction with the desired size (about 110 bp) was eluted electrophoretically.

#### Screening of rat FGF family DNA

The FGF family DNAs thus amplified by using FGF primers were inserted into pGEM-T DNA vector (Promega). The resultant recombinant vector was transformed into *E. coli* (strain XL1-blue), thereby obtaining DNA clones. DNA Sequencer 373A (Applied Biosystems, Inc.) was used for the analysis of cDNA sequence.

By determining the nucleotide sequences of all the DNA clones, besides cDNAs for FGF-3 and FGF-7 that were known, a novel FGF cDNA encoding a peptide with similar amino acid sequence to known FGF family peptides (about 50% similarity) was isolated. The clone was named FGF-10.

#### Structural analysis of entire coding region of FGF-10 cDNA

Primers were prepared based on the partial structure of FGF-10 cDNA identified in the above experiments. The entire coding region was obtained by using Rapid Amplification of cDNA Ends (RACE) method [Frohman, PCR Protocols - A Guide to Methods and Applications, Academic Press, pp. 28-38 (1990)]. Details are described under (1)-(6).



(1) Based on the partial structure of FGF-10 cDNA, primers A-D (Figure 2, sequence numbers 5, 6, 7 and 8) were prepared. In addition, primers X and Y were prepared by the RACE method (Figure 2, sequence numbers 9 and 10).

(2) Random hexaoligonucleotide was used as primer to synthesize cDNA with reverse transcriptase using the rat embryo mRNA as template. A poly(A) sequence was added to the 3'-terminus with 3'-deoxynucleotidyl transferase in the presence of deoxyadenine triphosphate. The cDNA thus prepared was used as template to perform PCR using primers B and X. Furthermore, primers A and Y were used to perform PCR. The resultant amplified fragments were inserted into pGEM-T, then transformed into *E. coli* (strain XL1-blue) thereby obtaining clones. The nucleotide sequences of several clones were determined to obtain a clone containing the above partial sequences. It was named pFGF-10 (5').

(3) cDNA was synthesized with reverse transcriptase using rat embryo mRNA as template and primer X. The cDNA thus produced was used as template to perform PCR using primers C and Y. Furthermore, primers D and Y were used to perform PCR. The resultant amplified fragments were inserted into pGEM-T, then transformed into *E. coli* (strain XL1-blue) thereby obtaining clones. The nucleotide sequences of several clones were determined to obtain a clone containing the above partial sequences. It was named pFGF-10 (3').

(4) Based on the most upstream nucleotide sequence of pFGF-10 (5') and the most downstream nucleotide sequence of pFGF-10 (3'), primers E and F, respectively, were prepared (Figure 2, sequence numbers 11 and 12).

(5) Rat embryo mRNA was used as template to synthesize single-stranded cDNA with reverse transcriptase using oligo(dT) as primer, which was then used as template to perform PCR using primers E and F. The resultant amplified fragments were inserted into pGEM-T, then transformed into *E. coli* (strain XL1-blue) thereby obtaining clones. The nucleotide sequences of several clones were determined thereby obtaining clones with a nucleotide sequence containing the most upstream nucleotide sequence of pFGF-10 (5') and the most downstream nucleotide sequence of pFGF-10 (3'). Among them, 1 clone was selected, named pFGF-10. FGF-10 cDNA containing entire coding region, carried by the plasmid, was analyzed.

(6) Taken together, the nucleotide sequence of sequence number 3 (804 bp) was determined.

#### Determination of entire amino acid sequence of rat FGF-10

(1) Based on the nucleotide sequence of FGF-10 cDNA obtained in the above experiments, it was known that the open reading frame of FGF-10 cDNA consists of 645 bp and that FGF-10 is a novel FGF consisting of 215 amino acids represented by sequence number 1.

(2) Analysis of the amino acid sequence revealed that it is a secreted protein with a signal sequence at the N-terminus. Mature protein is a polypeptide consisting of 179 amino acids between amino acid residue numbers 37-215. The Asn-Ser-Ser at 50-52 and Asn-Thr-Ser at 203-205 are N-glycosylation sites, suggesting that FGF-10 may be glycosylated.

#### Practical Example 2

##### Expression of rat FGF-10 in mammalian cells

##### Construction of plasmids

The plasmid pFGF-10 (Figure 3) was digested with SphI and PstI, then a fragment containing the full length cDNA was separated by polyacrylamide gel electrophoresis. The fragment was ligated into pUC19 that had been digested with SphI and PstI, then transformed into E. coli strain JM109, thereby obtaining the plasmid pUC-F10 containing FGF-10 cDNA. pUC-F10 was digested with HindIII and XbaI to cut off a fragment containing FGF-10 cDNA, that was then ligated into the mammalian cell expression vector pCDM8 that had been digested with HindIII and XbaI, followed by transformation into E. coli strain MC1061-/P3, thereby obtaining the plasmid pCDM8-F10SP containing FGF-10 cDNA downstream of CMV promoter.

On the other hand, since the nucleotide sequence upstream of the deduced translation start site in the FGF-10 cDNA was different from the Kozak consensus sequence, the translation efficiency of the mRNA was not considered to be high. Accordingly, to increase the translation efficiency, it was decided to perform mutation to convert the sequence upstream of the deduced translation start site to the Kozak consensus sequence [M. Kozak, The Journal of Cell Biology, Vol. 108, pages 229-241 (February, 1989)].

PCR was used to perform the mutation. pFGF-10 was used as template and a sense primer having, as shown in Figure 4, the HindIII site at the 5'-end and Kozak consensus sequence and an antisense having the XbaI site at the 5'-end were used (for reaction conditions, see Figure 4).

After the reaction, the PCR product was subjected to phenol-chloroform treatment, ether treatment then ethanol precipitation. After digestion with HindIII and XbaI, a fragment with about 700 bp was isolated by polyacrylamide gel electrophoresis. The fragment was ligated into the mammalian cell expression vector pCDM8 that had been digested with HindIII and XbaI. The vector was transformed into E. coli strain MC1061/P3. From the resultant colonies, 4 clones were selected and the nucleotide sequences were analyzed by using DNA Sequencer (Perkin Elmer model 373).

The results showed that in all the clones the sequence upstream of the deduced translation start site was converted to the Kozak consensus sequence, and that there was no mutation on the amino acid sequence encoded. One clone was selected from these clones, named pCDM8-F10HX.

### Practical Example 3

#### Transformation of rat FGF-10-expressing plasmids in COS-1 cells

The rat FGF-10-expressing plasmids constructed in Practical Example 2, pCDM8-F10SP and pCDM8-F10HX, were prepared in large quantities by standard procedures, and purified by performing cesium chloride density gradient centrifugation twice. The two plasmids and pCDM8 as control were transformed into COS-1 cells by electroporation. The transformed cells were cultured for 24 hours in DMEM containing 10% of bovine fetal serum that had been treated by lysine-Sepharose chromatography, then the medium was changed to serum-free DMEM. The culture was further performed for 96 hours. The culture supernatant thus obtained was centrifuged. The supernatant was stored at -80°C in aliquots.

#### Practical Example 4

##### Confirmation of expression of FGF-10 mRNA in cartilage by in situ hybridization

###### Preparation of probe

FGF-10 cDNA was inserted into the vector pGEM-T. The plasmid was transformed into *E. coli* strain JM-109. The bacteria were cultured in a large quantity. FGF-10 cDNA was highly purified by using Flexi Prep Kit from Pharmacia. The DNA sequence was confirmed by using Perkin Elmer 373A/DNA Sequencer. A cRNA probe was prepared by using DIG/RNA Labeling Kit (SP6/T7) from Boehringer.

###### Preparation of tissue slice

A 3 weeks old female Wistar rat was sacrificed. A thigh bone and tibia with the joint in its original shape was collected. After soft tissues were removed, it was trimmed into appropriate sizes then immediately soaked in fixing solution (4% paraformaldehyde). After fixing at 4°C overnight, dehydration was performed, then it was soaked in ash-removing solution (10% EDTA and 15% glycerol in PBS) for 4-5 days (the solution was replaced with fresh solution every day). The knee joint was trimmed to a thickness of about 2 cm, and was then soaked in OTC compound and frozen with liquid nitrogen. A cryostat was used to obtain 10  $\mu$ m thick joint tissue slices, which were then mounted on a silane-coated slide glass.

###### Hybridization

After the above joint tissue slides were subjected to pretreatment (digestion with proteinase K, inactivation of endogenous alkaline phosphatase with 0.2 M HCl, and acetylation with 0.1 M TEA and 0.25% acetic anhydride), they were dehydrated with ethanol. The above probe was diluted tenfold with hybridization solution (50% formamide, 10 mM Tris-HCl/pH 7.6, 200  $\mu$ g/mL tRNA, 1 x Denhardt's solution, 10% Dextran sulfate, 600 mM NaCl, and 0.25% SDS), then 50  $\mu$ L per slide was used. The sample was covered with a small sheet of parafilm. The hybridization was performed at 50°C for 16 hours. Excess probe was digested with RNase A. After washed with SSC, antibody binding and color development were performed.

###### Antibody binding and color development

After the probe was washed off, the slide was soaked in blocking solution for 60 min. Alkaline phosphatase-labeled anti-digoxigenin antibody (anti-digoxigenin-AP:Fab fragment,

from Boehringer-Mannheim) was added onto the slide. After incubation at 37°C for 1 hour, the antibody solution was washed off. NBT.X(?) -phosphate was added, followed by incubation at 37°C for color development (12 hours). After the color was developed, the slide was soaked in color development stopping solution (10 mM Tris-HCl/pH 7.6, 1 mM EDTA/pH 8.0). After washed with distilled water, the slide was sealed with water.

## Results

As shown in Figure 5 (A) and (B), color development was seen in chondral cells. Since FGF-10 mRNA is expressed in chondral cells, it is suggested that FGF-10 may be a factor involved in wound healing in bone and cartilage.

## Practical Example 5

### Evaluation of cell-growth-promoting activity in FRSK cells

#### Cell culture

The rat epithelial cell line FRSK was cultured in culture flask with a culture area of 75 cm<sup>2</sup> in 15 mL of F-12 medium containing 10% of bovine fetal serum at 37°C in an atmosphere of 5% carbon dioxide/95% air. The cells were split at 1/10 once a week.

#### Expression of FGF-10 protein

FGF-10 was transiently expressed in COS-1 cells (see Practical Example 3). The culture supernatant was used in the following assays (in the following, the culture supernatants obtained from pCDM8-F10SP, pCDM8-F10HX and the control pCDM8 are represented by FGF-10/Sp, FGF-10/Hx and Bq, respectively).

DNA synthesis assay (tritium-labeled thymidine incorporation): The cells were cultured until sub-confluence, then collected by trypsin treatment. A cell suspension at 10,000 cells/mL in the above medium was prepared, then distributed at 100  $\mu$ L/well in 96-well plate. The plate was incubated at 37°C in an atmosphere of 5% carbon dioxide/95% air. The medium was replaced with 100  $\mu$ L of fresh medium once every 2 days. After 7 days of culture, the medium was replaced with 100  $\mu$ L of F-12 medium containing 0.1% of bovine fetal serum. After 24 hours, 25  $\mu$ L of the COS supernatant was added. After 18 hours culture at 37°C in an atmosphere of 5% carbon dioxide/95% air, 20  $\mu$ L of F-12 medium containing 0.2  $\mu$ Ci of tritium-labeled thymidine was added, followed by incubation under the same conditions. After 4 hours, the medium was removed, and 50  $\mu$ L of 2 N

NaOH was added, followed by standing for 30 min to kill the cells. After neutralizing with 1 N HCl, the cells were recovered with a cell harvester, and counted in Betaplate.

## Results

As shown in Figure 6, compared to the control (Bq, 100%), the FGF-10 expression samples (Sp and Hx) both greatly enhanced the incorporation of tritium-labeled thymidine into FRSK (286% and 501 %, respectively). Thus, it is shown that FGF-10 is a factor promoting the growth of epithelial cells.

### Practical Example 6

#### Structural analysis of human FGF-10 gene

##### Preparation of human DNA library

Commercially available human lung poly(A) RNA (Clontech, Cat. No. 6524, from the whole lung of a male adult) was used as template to prepare human lung cDNA using random primer (6 mer) as primer and Moloney murine leukemia virus reverse transcriptase. Specifically, human lung poly(A) RNA (5  $\mu$ g) was incubated in a solution containing 300 units of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL), 15 units of human placenta RNase inhibitor (Wako Pure Chemicals) and 0.5  $\mu$ g of random primer (6 mer) at 37°C for 60 min, thereby obtaining the cDNA.

##### Preparation of primers for amplifying human FGF-10 DNA and amplification of human FGF family DNAs

The 2 FGF primers used in Practical Example 1 shown in Figure 1 (Tyr-Leu-Ala-Met-Asn-Lys and Tyr-Asn-Thr-Tyr-Ala-Ser) were selected and used for the amplification of human FGF-10 DNA.

Human lung cDNA was used as template. The above 2 FGF primers and Taq DNA polymerase were used to amplify FGF family DNAs by the polymerase chain reaction (PCR) method. Specifically, a reaction solution (25  $\mu$ L) containing an appropriate amount of cDNA, 0.05 unit/ $\mu$ L of Taq DNA polymerase (Wako Pure Chemicals) and 5 pmol/ $\mu$ L of the above sense or antisense primer was subjected to 30 cycles of PCR. After the reaction, the solution was applied to 8% polyacrylamide gel electrophoresis, and the fraction with the desired size (about 110 bp) was eluted electrophoretically.

### Screening of human FGF family DNAs

The FGF family DNAs thus amplified by using FGF primers were inserted into pGEM-T DNA vector (Promega). The resultant recombinant vector was transformed into *E. coli* (strain XL1-blue), thereby obtaining DNA clones. DNA Sequencer 373A (Applied Biosystems, Inc.) was used for the analysis of the cDNA sequence.

By determining the nucleotide sequences of all the DNA clones, a cDNA encoding a peptide identical to that of rat FGF-10 was identified. This DNA is considered to encode the human FGF-10.

### Structural analysis of entire coding region of human FGF-10 cDNA

A cDNA containing the entire coding region of human FGF-10 was amplified and analyzed as in Practical Example 1, thereby obtaining the nucleotide sequence represented by sequence number 4. Details are described under items (1)-(6) below.

(1) Based on the partial structure of human FGF-10 cDNA, primers A' and D' (Figure 2, sequence numbers 13 and 14) were prepared. Primers B, C, X and Y were identical to those in Practical Example 1 (Figure 2, sequence numbers 5, 6, 7 and 8).

(2) Random hexaoligonucleotide was used as primer to synthesize cDNA with reverse transcriptase using the human lung mRNA as template. A poly(A) sequence was added to the 3'-terminus with 3'-deoxynucleotidyl transferase in the presence of deoxyadenine triphosphate. The cDNA thus prepared was used as template to perform PCR using primers A' and X. Furthermore, primers B and Y were used to perform PCR. The resultant amplified fragments were inserted into pGEM-T, then transformed into *E. coli* (strain XL1-blue) thereby obtaining a clone containing portions of the above partial sequences. The clone was named phFGF-10 (5').

(3) cDNA was synthesized with reverse transcriptase using human lung mRNA as template and primer X. The cDNA thus produced was used as template to perform PCR using primers C and X. Furthermore, primers D' and Y were used to perform PCR. The resultant amplified fragments were inserted into pGEM-T, then transformed into *E. coli* (strain XL1-blue) thereby obtaining clones. The nucleotide sequences of several clones were

determined to obtain a clone containing portions of the above partial sequence. It was named phFGF-10 (3').

(4) Since the nucleotide sequence of phFGF-10 (5') upstream of the translation region was also present in the rat gene, primer E (Figure 2, sequence number 12) was used as primer for the 5'-end. On the other hand, based on the most downstream nucleotide sequence of phFGF-10 (3') primer F' was prepared as primer for the 3'-end (Figure 2, sequence number 15).

(5) Human lung mRNA was used as template to synthesize single-stranded cDNA with reverse transcriptase using oligo(dT) as primer, which was then used as template to perform PCR using primers E and F'. The resultant amplified fragments were inserted into pGEM-T, then transformed into E. coli (strain XL1-blue) thereby obtaining clones. The nucleotide sequences of several clones were determined thereby obtaining clones with a nucleotide sequence containing the most upstream nucleotide sequence of phFGF-10 (5') and the most downstream nucleotide sequence of phFGF-10 (3'). Among them, 1 clone was selected, named phFGF-10. Human FGF-10 cDNA containing the entire coding region, carried by the plasmid, was analyzed.

(6) Taken together, the nucleotide sequence of sequence number 4 (690 bp) was determined.

#### Determination of entire amino acid sequence of human FGF-10

(1) Based on the nucleotide sequence of the cDNA obtained in the above experiments, it was known that the open reading frame of human FGF-10 cDNA consists of 624 bp and that human FGF-10 is a polypeptide consisting of 208 amino acids represented by sequence number 2. Analysis of the amino acid sequence revealed that it is a secreted protein with a signal sequence at the N-terminus. The mature protein is a polypeptide consisting of 171 amino acids between amino acid residue numbers 38-208. The Asn-Ser-Ser at 51-53 and Asn-Thr-Ser at 196-198 are N-glycosylation sites, suggesting that the protein may be glycosylated.



## Practical Example 7

### Expression and purification of mature protein of human FGF-10

pFGF-10 was used as template to perform 15 cycles of PCR using the following primer pair (sequence numbers 16 and 17), then phenol-chloroform treatment and ethanol precipitation were performed. After digestion with NdeI and BamHI and subsequent polyacrylamide gel electrophoresis, a band with the targeted size was isolated, thereby obtaining a DNA fragment (a) corresponding to the amino acid sequence of the mature protein of human FGF-10 cDNA. On the other hand, the E. coli expression vector pET11c (Stratagen) was digested with NdeI and BamHI, then the vector DNA (b) was obtained by agarose gel electrophoresis. (a) and (b) were ligated to each other, then transformed into E. coli strain JM109, thereby obtaining clones. Among the clones, a plasmid with (a) in the right orientation was isolated and the DNA sequence was determined and named pET-hFGF-10. The plasmid was transformed into E. coli BL21 (DE3). One of the resultant recombinant clones was named BL21 (DE3)/pET-hFGF-10, which was used for the production of human FGF-10.

Four flasks of the bacteria containing BL21 (DE3)/pET-hFGF-10 were cultured in 10 mL each of LB medium containing 100 µg/mL of ampicillin at 37°C overnight. On the next day, the entire contents of each flask was added to 500 mL of TB medium containing 100 µg/mL of (ampicillin), and the mixture was cultured at 37°C with agitation. After the OD600 reached 0.8, IPTG was added to a final concentration of 1 mM. The culture temperature was decreased to 28°C and the culture was continued for 6 hours.

The culture medium was separated by centrifugation. The resultant bacteria were washed once with 50 mM Tris-HCl, pH 8.0, then suspended in 50 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 2 µg/mL leupeptin, 2 µg/mL pepstatin and 1 mM PMSF. The bacteria were broken by sonication, then centrifuged in Beckman J2-21M/E high speed refrigerated centrifuge with JA-20 rotor at 15000 rpm for 1 hour. The supernatant was collected. HiTrap Heparin (5 mL, from Pharmacia) was equilibrated with 50 mM Tris-HCl, pH 8.0, then the above supernatant was applied. The resin was washed with 50 mM Tris-HCl, pH 8.0 until A260 of the eluate returned to the baseline. A continuous NaCl concentration gradient up to 3 M was applied to elute proteins. A protein with a molecular weight of about 19 kDa, that looked to be the recombinant human FGF-10, was eluted at about 1.2 M NaCl. The flow rate was 2 mL/min.

Subsequently, the above eluate was diluted 2-fold with 50 mM Tris-HCl, pH 8.0, then applied to Hi Trap SP (5 mL, from Pharmacia). After washing with 50 mM Tris-HCl, pH 8.0, a continuous NaCl concentration gradient up to 2 M was applied to elute proteins. A protein with a molecular weight of about 19 KDa, that seemed to be the recombinant human FGF-10, was eluted at about 1.2 M NaCl. The flow rate was 2 mL/min. The above eluate was dialyzed against PBS (-), then Pyrosep 1C (from Daicel Kogyo) was added in an amount of 1/10 in order to remove endotoxin. After agitation at 4°C for 2 hours, the supernatant was recovered. The endotoxin was assayed by using Endospecy(?) ES-6 (Seikagaku Kogyo), and the level was below detection limit. Protein was assayed by using Protein Assay Kit (Bio Rad), and a total of 3.5 mg of protein was obtained.

#### Practical Example 8

##### Effects of FGF-10 on bone tissues

In the following, the pharmaceutical preparation of the bone (cartilage) disease-treating agent and the effects on bone (cartilage) tissue genesis and repair are described by the way of pharmaceutical preparation and testing examples.

**Pharmaceutical Preparation:** The purified mature protein of FGF-10 produced in Practical Example 7 was prepared as an aqueous solution for injection. Specifically, human FGF-10 (2.12 mg) was dissolved in saline (1 mL), then used for the following experiments.

##### In vivo evaluation of efficacy: effects of FGF-10 on bone tissue

The aqueous solution of FGF-10 prepared in Pharmaceutical Preparation Example 1 was injected intramedullarily at the tibia using microsyringe with 27 G needle in 3 groups of 4 weeks old male Wistar rats (body weight: 94-120 g), each 3-4 animals, after anesthetized with ether, at doses of 10.6, 21.2 and 0 (as control)  $\mu$ g.

After 4 days, the animals were sacrificed, and the tibias were removed. Soft X-ray photographs of the tibias were taken, and the photos were input into a personal computer by using scanner. An image processing program was used to observe images of osteogenesis.

The results of FGF-10 and control groups are shown in Figure 7 and Figure 8, respectively. In the human FGF-10 administration groups, increase in osteogenesis was

recognized. The results of intramedullary osteogenesis on day 4 after human FGF-10 administration, evaluated by image analysis of soft X-ray photos, are shown in Table 1.

Table 1

FGF-10 ( $\mu$ g)	0	10.6	21.2
number of animals	3	4	4
number of animals which showed osteogenesis compared to control animals	--	4	4

These results show that human FGF-10 has genesis and repair-promoting activity for bone/cartilage tissues, which is important for the treatment of bone/cartilage diseases.

### Practical Example 9

#### Preparation of FGF-10 mutants: construction of FGF-10 mutant plasmids expressable in mammalian cells

##### Construction of human FGF-10 plasmid expressable in mammalian cells

The plasmid was constructed as for pCDM8-F10HX described under Practical Example 2. Thus, the plasmid phFGF-10 containing natural human FGF-10 sequence was used as template to perform PCR using the following primer hF10HX instead of the primer F10HS shown in Figure 4 and the following primer hF10XR instead of the primer F10XR. The product was digested with HindIII and XbaI, then a fragment of about 700 bp was isolated by polyacrylamide gel electrophoresis. The fragment was ligated with pCDM8 that had been digested with HindIII and XbaI. The plasmid was transformed into E. coli strain MC1061/p3, thereby obtaining the targeted plasmid pCDM8-hF10HX expressable in mammalian cells.

Primers:                      5'      →      3'

h F 1 0 H S      TTTAAGCTTCCACCATGTGGAAATGGATACTGAC

h F 1 0 X R      TTTTCTAGAACAAACGGTGCCTTCCTCTATG

#### Construction of pCDM8-F10 (S106) (Figure 9A)

First, primer 1 that has the desired mutation, primer 2 that is partially complementary to primer 1, and primer 3 and primer 4 that anneal to regions outside the region between the above 2 primers, were prepared. The plasmid pCDM8-hF10HX containing natural human FGF-10 sequence was used as template to perform a PCR using primers 1 and 3, thereby obtaining DNA fragment 1 that has the mutation. On the other hand, a PCR was performed using the same template and primers 2 and 4, thereby obtaining DNA fragment 2 that had the same mutations and partially overlapped with DNA fragment 1. Then, DNA fragments 1 and 2 thus generated were used as templates to perform a PCR using primers 3 and 4, thereby obtaining DNA fragment 3. Subsequently, DNA fragment 3 was digested with the restriction enzymes HindIII and XbaI, then ligated with pCDM8-hF10HX that had been digested with the same restriction enzymes and dephosphorylated. The plasmid was transformed into *E. coli* strain MC1061/p3, thereby obtaining the plasmid pCDM8-F10 (S106) that was able to express the protein with the desired mutation.

#### Construction of pCDM8-F10 (S150)

By 2 step PCR as for the construction of pCDM8-F10 (S106) except that primers 5 and 6 were used instead of primers 1 and 2, respectively, a DNA fragment with the desired mutation was obtained. The DNA was digested with restriction enzymes HindIII and XbaI, then ligated with pCDM8-hF10HX that had been digested with the same restriction enzymes and dephosphorylated. The plasmid was transformed into *E. coli* strain MC1061/p3, thereby obtaining the plasmid pCDM8-F10 (S150) that was able to express the protein with the desired mutation.

#### Construction of pCDM8-F10 (A51)

By 2 step PCR as for the construction of pCDM8-F10 (S106) except that primers 7 and 8 were used instead of primers 1 and 2, respectively, a DNA fragment with the desired mutation was obtained. The DNA was digested with the restriction enzymes HindIII and XbaI, then ligated with pCDM8-hF10HX that had been digested with the same restriction enzymes and dephosphorylated. The plasmid was transformed into *E. coli* strain MC1061/p3, thereby obtaining the plasmid pCDM8-F10 (A51) that was able to express the protein with the desired mutation.

#### Construction of pCDM8-F10 (A196)

By 2 step PCR as for the construction of pCDM8-F10 (S106) except that primers 9 and 10 were used instead of primers 1 and 2, respectively, a DNA fragment with the desired mutation was obtained. The DNA was digested with the restriction enzymes HindIII and XbaI, then ligated with pCDM8-hF10HX that had been digested with the same restriction enzymes and dephosphorylated. The plasmid was transformed into E. coli strain MC1061/p3, thereby obtaining the plasmid pCDM8-F10 (A196) that was able to express the protein with the desired mutation.

#### Construction of pCDM8-F10 (C84)

By 2 step PCR as for the construction of pCDM8-F10 (S106) except that primers 11 and 12 were used instead of primers 1 and 2, respectively, a DNA fragment with the desired mutation was obtained. The DNA was digested with the restriction enzymes HindIII and XbaI, then ligated with pCDM8-hF10HX that had been digested with the same restriction enzymes and dephosphorylated. The plasmid was transformed into E. coli strain MC1061/p3, thereby obtaining the plasmid pCDM8-F10 (C84) that was able to express the protein with the desired mutation.

#### Construction of pCDM8-F10 (S150/S106)

By the digestion of pCDM8-F10 (S106) with HindIII and MvaI, a DNA fragment of about 340 bp containing the S106 mutation was obtained. On the other hand, pCDM8-F10 (S150) was digested with MvaI and XbaI, thereby obtaining a DNA fragment of about 320 bp containing the S150 mutation. These 2 DNA fragments were ligated with pCDM8-hF10HX that had been digested with the restriction enzymes HindIII and XbaI and dephosphorylated. The plasmid was transformed into E. coli strain MC1061/p3, thereby obtaining the plasmid pCDM8-F10 (S150/S106) that was able to express the protein with the desired mutations.

#### Construction of pCDM8-F10 (C84/S106)

By the digestion of pCDM8-F10 (C84) with HindIII and ScaI, a DNA fragment of about 270 bp containing the C84 mutation was obtained. On the other hand, pCDM8-F10 (S106) was digested with the restriction enzymes ScaI and XbaI, thereby obtaining a DNA fragment of about 390 bp containing the S106 mutation. These 2 DNA fragments were ligated with pCDM8-hF10HX that had been digested with the restriction enzymes HindIII

and XbaI and dephosphorylated. The plasmid was transformed into E. coli strain MC1061/p3, thereby obtaining the plasmid pCDM8-F10 (C84/S106) that was able to express the protein with desired mutations.

#### Construction of pCDM8-F10 (A51/A196)

By the digestion of pCDM8-F10 (A51) with HindIII and ScaI, a DNA fragment of about 270 bp containing the A51 mutation was obtained. On the other hand, pCDM8-F10 (A196) was digested with the restriction enzymes ScaI and XbaI, thereby obtaining a DNA fragment of about 390 bp containing the A196 mutation. These 2 DNA fragments were ligated with pCDM8-hF10HX that had been digested with the restriction enzymes HindIII and XbaI and dephosphorylated. The plasmid was transformed into E. coli strain MC1061/p3, thereby obtaining the plasmid pCDM8-F10 (A51/A196) that was able to express the protein with the desired mutations.

#### Construction of pCDM8-F10 (DN1) (Figure 9B)

Primer 13 that was complementary to the region upstream of the deleted region and primer 14 that was complementary to primer 13 were prepared. The plasmid pCDM8-hF10HX was used as template to perform a PCR using primers 13 and 3, thereby obtaining DNA fragment 4. On the other hand, a PCR was performed using the same template and primers 14 and 4, thereby obtaining DNA fragment 5. Then, DNA fragments 4 and 5 thus generated were used as templates to perform a PCR using primers 3 and 4, thereby obtaining DNA fragment 6 with the desired deletion. Subsequently, DNA fragment 6 was digested with the restriction enzymes HindIII and XbaI, then ligated with pCDM8-hF10HX that had been digested with the same restriction enzymes and dephosphorylated. The plasmid was transformed into E. coli strain MC1061/p3, thereby obtaining the plasmid pCDM8-F10 (DN1) that was able to express the protein with the desired mutation.

#### Construction of pCDM8-F10 (DN2)

By 2 step PCR as for the construction of pCDM8-F10 (DN1) except that primers 15 and 16 were used instead of primers 13 and 14, respectively, a DNA fragment with the desired deletion was obtained. The DNA was digested with the restriction enzymes HindIII and XbaI, then ligated with pCDM8-hF10HX that had been digested with the same restriction enzymes and dephosphorylated. The plasmid was transformed into E. coli strain

MC1061/p3, thereby obtaining the plasmid pCDM8-F10 (DN2) that was able to express the protein with the desired mutation.

#### Construction of pCDM8-F10 (DN3)

By 2 step PCR as for the construction of pCDM8-F10 (DN1) except that primers 17 and 18 were used instead of primers 13 and 14, respectively, a DNA fragment with the desired deletion was obtained. The DNA was digested with the restriction enzymes HindIII and XbaI, then ligated with pCDM8-hF10HX that had been digested with the same restriction enzymes and dephosphorylated. The plasmid was transformed into E. coli strain MC1061/p3, thereby obtaining the plasmid pCDM8-F10 (DN3) that was able to express the protein with the desired mutation.

#### Construction of pCDM8-F10 (DN4)

By 2 step PCR as for the construction of pCDM8-F10 (DN1) except that primers 19 and 20 were used instead of primers 13 and 14, respectively, a DNA fragment with the desired deletion was obtained. The DNA was digested with the restriction enzymes HindIII and XbaI, then ligated with pCDM8-hF10HX that had been digested with the same restriction enzymes and dephosphorylated. The plasmid was transformed into E. coli strain MC1061/p3, thereby obtaining the plasmid pCDM8-F10 (DN4) that was able to express the protein with the desired mutation.

#### Construction of pCDM8-F10 (DN5)

By 2 step PCR as for the construction of pCDM8-F10 (DN1) except that primers 21 and 22 were used instead of primers 13 and 14, respectively, a DNA fragment with the desired deletion was obtained. The DNA was digested with the restriction enzymes HindIII and XbaI, then ligated with pCDM8-hF10HX that had been digested with the same restriction enzymes and dephosphorylated. The plasmid was transformed into E. coli strain MC1061/p3, thereby obtaining the plasmid pCDM8-F10 (DN5) that was able to express the protein with the desired mutation.

#### Construction of pCDM8-F10 (DC1) (Figure 9C)

Primer 23 that had the codon for Asn-196 replaced by the stop codon and had an XbaI site was prepared. The plasmid pCDM8-hF10HX was used as template to perform a PCR using primers 13 and 4, thereby obtaining DNA fragment 7. Then, DNA fragment 7

was digested with the restriction enzymes HindIII and XbaI, then ligated with pCDM8-hF10HX that had been digested with the same restriction enzymes and dephosphorylated. The plasmid was transformed into E. coli strain MC1061/p3, thereby obtaining the plasmid pCDM8-F10 (DC1) that was able to express the protein with the desired mutation.

#### Construction of pCDM8-F10 (DC2)

By 1 step PCR as for the construction of pCDM8-F10 (DC1) except that primer 24 was used instead of primer 23, a DNA fragment with the desired deletion was obtained. The DNA was digested with the restriction enzymes HindIII and XbaI, then ligated with pCDM8-hF10HX that had been digested with the same restriction enzymes and dephosphorylated. The plasmid was transformed into E. coli strain MC1061/p3, thereby obtaining the plasmid pCDM8-F10 (DC2) that was able to express the protein with the desired mutation.

#### Construction of pCDM8-F10 (DC3)

By 1 step PCR as for the construction of pCDM8-F10 (DC1) except that primer 25 was used instead of primer 23, a DNA fragment with the desired deletion was obtained. The DNA was digested with the restriction enzymes HindIII and XbaI, then ligated with pCDM8-hF10HX that had been digested with the same restriction enzymes and dephosphorylated. The plasmid was transformed into E. coli strain MC1061/p3, thereby obtaining the plasmid pCDM8-F10 (DC3) that was able to express the protein with the desired mutation.

#### Construction of pCDM8-F10 (DC4)

By 1 step PCR as for the construction of pCDM8-F10 (DC1) except that primer 26 was used instead of primer 23, a DNA fragment with the desired deletion was obtained. The DNA was digested with the restriction enzymes HindIII and XbaI, then ligated with pCDM8-hF10HX that had been digested with the same restriction enzymes and dephosphorylated. The plasmid was transformed into E. coli strain MC1061/p3, thereby obtaining the plasmid pCDM8-F10 (DC4) that was able to express the protein with the desired mutation.

#### Construction of pCDM8-F10 (SA) (Figure 9D)

Four primers (SA1F, SA1R, SA2F and SA2R) had the desired mutations were prepared. The 5'-ends of these oligonucleotides were phosphorylated, then SA1F and SA1R and SA2F and SA2R were annealed together, thereby obtaining SA1F/SA1R and SA2F/-SA2R fragments. These 2 fragments were digested with HindIII and BstXI, then ligated with



pCDM8-hF10HX that had been digested with the same restriction enzymes and dephosphorylated. The plasmid was transformed into *E. coli* strain MC1061/p3, thereby obtaining the plasmid pCDM8-F10 (SA) that was able to express the protein with the desired mutation.

5' → 3'

primer - 1 GAAGGAGAACAGCCCGTACAGCATCCTG  
primer - 2 TGCTGTACGGGCTGTTCTCCTTCTTGGT  
primer - 3 GATCCTCTAGAAGAAACGGT  
primer - 4 GTAATACGACTCACTATAGGGC  
primer - 5 TAACAATGACAGTAAGCTGAAGGAGAGG  
primer - 6 CCTTCAGCTTACTGTCATTGTTAAATTC  
primer - 7 AGAGGCCACCGCCTCTTCTCCTCCTCC  
primer - 8 AGGAAGAAGAGGCGGTGGCCTCTGGTGA  
primer - 9 ACGAAGGAAAGCCACCTCTGCTCACTTT  
primer - 10 GAGCAGAGGTGGCTTTCCTTCGTGTTTT  
primer - 11 AAAGCTATTCTGTTTCACCAAGTACTTT  
primer - 12 ACTTGGTGAAACAGAATAGCTTTCTCCA  
primer - 13 CTGCCAAGCCAACTCTTCTCCTCCTCCTT  
primer - 14 AAGAAGAGTTGGCTTGGCAGGTGACAGGGA  
primer - 15 CTGCCAAGCCCGGGGAAGGCATGTGCCGAG  
primer - 16 GCCTTCCCGCGGCTTGGCAGGTGACAGGGA  
primer - 17 CTGCCAAGCCCTATTCTCTTTCACCAAGTA  
primer - 18 AAGAGAATAGGGCTTGGCAGGTGACAGGGA  
primer - 19 CTGCCAAGCCGAGAACTGCCCCGTACAGCAT  
primer - 20 GGCAGTTCTCGGCTTGGCAGGTGACAGGGA  
primer - 21 CTGCCAAGCCGGGAACTCTATGCCTCAAA  
primer - 22 AGAGTTTCCCGGCTTGGCAGGTGACAGGGA  
primer - 23 TTTTCTAGACTATTTCTTCGTGTTTT  
primer - 24 TTTTCTAGACTATCTCCTTGGAGCTCC  
primer - 25 TTTTCTAGACTACTTCAGCTTACAGTC  
primer - 26 TTTTCTAGACTACTTCTTGTTCATGGC

S A 1 F AGCTTCCACCATGTGGAAATGCATACTGACACATGCTGCCTCAGCCTTTCCTCA  
CCTGCCTGGCGCTG

S A 1 R CAGGTGAGGAAAGGCTGAGGCAGCATGTGTCAGTATCCATTTCCACATGGTGA

S A 2 F CTGCCGCTGCCTTTTTGTTGCTGTTCTTGGTGTCTTCCGTACCTGTCACCTGCC  
AAGCCC

S A 2 R TTGGCAGGTGACAGGTACGGAAGACACCAACAACAGCAACAAAAAGGCAGCCGC  
AGCAGCGCCAGG

### Transformation of COS-1 cells with FGF-10 mutant-expressing plasmids

The above 17 FGF-10 mutant-expressing plasmid DNAs were prepared in large quantities by standard procedures, and purified by performing cesium chloride density gradient centrifugation twice. The 17 plasmids and pCDM8-hF10HX and pCDM8 as controls were transformed into COS-1 cells by electroporation. The transformed cells were cultured for 24 hours in DMEM or IMEM containing 10% of bovine fetal serum, then the medium was changed to serum-free IMEM containing 10  $\mu$ g/mL of heparin. The culture was further performed for 96 hours. The culture supernatant thus obtained was centrifuged. The supernatant thus collected was diluted tenfold with IMEM containing 10  $\mu$ g/mL of heparin and was stored at -80°C in aliquots.

### Evaluation of cell growth-promoting activity in FRSK cells

Results: As shown in Figure 10, all the culture supernatant samples obtained from the FGF-10 mutant (DN1, DN2, DC2, SA, A51, A196, S150, S106, C84/S106, S150/S106 and A51/A196)-expressing plasmids significantly promoted the incorporation of tritium-labeled thymidine, compared to the culture supernatant sample obtained from pCDM8.

### Possible Applications in the Industry

As clearly shown by the above results, human FGF-10 was found to have excellent action of generation and regeneration of bone/cartilage tissues and be useful for the treatment of diseases of bone/cartilage tissues. Thus, the present therapeutic agent for diseases of bone/cartilage is useful for the treatment of various diseases of bone/cartilage tissues, for example, (1) the repair of damaged cartilage caused by deforming arthritis or arthritis as a result of autoimmune diseases such as chronic rheumatoid arthritis, etc., (2) the repair of damaged cartilage caused by traumas or osteochondritis dissecans or the promotion of chondrogenesis after osteotomy, (5) the repair after fractures, (6) the repair after bone damage, (7) the promotion of osteogenesis at sites where local bone reduction due to osteoporosis, etc. has been detected.

Thus, the present invention provides pharmaceutical preparations using the novel factor, as well as the DNA encoding FGF-10, the expression vectors carrying the DNA, the transformants, the method for producing the recombinant proteins using the above reagents, and the recombinant proteins.

# Sequence Table

sequence number 1  
sequence length: 215  
sequence form: amino acid  
topology: linear chain  
sequence type: peptide  
origin  
species name: rat  
sequence

Met	Trp	Lys	Trp	Ile	Leu	Thr	His	Cys	Ala	Ser	Ala	Phe	Pro	His	Leu
1				5					10					15	
Pro	Gly	Cys	Cys	Cys	Cys	Phe	Leu	Leu	Leu	Phe	Leu	Val	Ser	Ser	Val
			20					25					30		
Pro	Val	Thr	Cys	Gln	Ala	Leu	Gly	Gln	Asp	Met	Val	Ser	Pro	Glu	Ala
		35					40					45			
Thr	Asn	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Phe
	50					55						60			
Ser	Ser	Pro	Ser	Ser	Ala	Gly	Arg	His	Val	Arg	Ser	Tyr	Asn	His	Leu
65				70						75				80	
Gln	Gly	Asp	Val	Arg	Trp	Arg	Lys	Leu	Phe	Ser	Phe	Thr	Lys	Tyr	Phe
			85					90					95		
Leu	Lys	Ile	Glu	Lys	Asn	Gly	Lys	Val	Ser	Gly	Thr	Lys	Lys	Glu	Asn
		100					105					110			
Cys	Pro	Tyr	Ser	Ile	Leu	Glu	Ile	Thr	Ser	Val	Glu	Ile	Gly	Val	Val
		115					120					125			
Ala	Val	Lys	Ala	Ile	Asn	Ser	Asn	Tyr	Tyr	Leu	Ala	Met	Asn	Lys	Lys
	130					135					140				
Gly	Lys	Leu	Tyr	Gly	Ser	Lys	Glu	Phe	Asn	Asn	Asp	Cys	Lys	Leu	Lys
145				150						155				160	
Glu	Arg	Ile	Glu	Glu	Asn	Gly	Tyr	Asn	Thr	Tyr	Ala	Ser	Phe	Asn	Trp
			165					170					175		
Gln	His	Asn	Gly	Arg	Gln	Met	Tyr	Val	Ala	Leu	Asn	Gly	Lys	Gly	Ala
		180					185					190			
Pro	Arg	Arg	Gly	Gln	Lys	Thr	Arg	Arg	Lys	Asn	Thr	Ser	Ala	His	Phe
		195					200					205			
Leu	Pro	Met	Val	Val	His	Ser									
	210					215									

sequence number 2  
sequence length: 208  
sequence form: amino acid  
topology: linear chain  
sequence type: peptide  
origin  
species name: human  
sequence

Met	Trp	Lys	Trp	Ile	Leu	Thr	His	Cys	Ala	Ser	Ala	Phe	Pro	His	Leu
1				5					10				15		
Pro	Gly	Cys	Cys	Cys	Cys	Cys	Phe	Leu	Leu	Leu	Phe	Leu	Val	Ser	Ser
				20				25					30		
Val	Pro	Val	Thr	Cys	Gln	Ala	Leu	Gly	Gln	Asp	Met	Val	Ser	Pro	Glu
				35				40					45		
Ala	Thr	Asn	Ser	Ser	Ser	Ser	Ser	Phe	Ser	Ser	Pro	Ser	Ser	Ala	Gly
				50				55					60		
Arg	Ile	Val	Arg	Ser	Tyr	Asn	His	Leu	Gln	Gly	Asp	Val	Arg	Trp	Arg
65						70					75				80
Lys	Leu	Phe	Ser	Phe	Thr	Lys	Tyr	Phe	Leu	Lys	Ile	Glu	Lys	Asn	Gly
				85						90				95	
Lys	Val	Ser	Gly	Thr	Lys	Lys	Glu	Asn	Cys	Pro	Tyr	Ser	Ile	Leu	Glu
				100						105				110	
Ile	Thr	Ser	Val	Glu	Ile	Gly	Val	Val	Ala	Val	Lys	Ala	Ile	Asn	Ser
				115						120				125	
Asn	Tyr	Tyr	Leu	Ala	Met	Asn	Lys	Lys	Gly	Lys	Leu	Tyr	Gly	Ser	Lys
				130						135				140	
Glu	Phe	Asn	Asn	Asp	Cys	Lys	Leu	Lys	Glu	Arg	Ile	Glu	Glu	Asn	Gly
145						150					155				160
Tyr	Asn	Thr	Tyr	Ala	Ser	Phe	Asn	Trp	Gln	His	Asn	Gly	Arg	Gln	Met
				165							170				175
Tyr	Val	Ala	Leu	Asn	Gly	Lys	Gly	Ala	Pro	Arg	Arg	Gly	Gln	Lys	Thr
				180							185				190
Arg	Arg	Lys	Asn	Thr	Ser	Ala	His	Phe	Leu	Pro	Met	Val	Val	His	Ser
				195							200				205

sequence number 3  
sequence length: 804 bp  
sequence form: nucleic acid  
topology: double-stranded chain  
sequence type: cDNA  
origin  
species name: rat  
existing position: 109-753  
method for determining the characteristics: E  
sequence

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TAACCACTAG CCATCACCTC CAGCTGTCTC TTGCGCTCGC ACCAGGTCTT ACCCTTCCAG   60
TATGTTCCCTT CTGATCAGAC AATTTCAGT GCCGAGAGTT TCAGTACA ATG TCG AAG   117
TGG ATA CTG ACA CAT TGT GCC TCA GCC TTT CCC CAC CTG CCG GGC TGC   165
TGT TGC TGC TTC TTG TTG CTC TTC TTG GTG TCT TCC GTC CCT GTC ACC   213
TGC CAA GCT CTT GGT CAG GAC ATG GTG TCA CCG GAG GCT ACC AAC TCC   261
TCT TCC TCC TCC TCT TCC TCC TCC TCG TCC TCT TCC TTC TCC TCT CCT   309
TCC AGC GCG GGG AGG CAT GTG CCG AGC TAC AAT CAC CTC CAG GGA GAT   357
GTC CCG TGG AGA AAG CTG TTC TCC TTC ACC AAG TAC TTT CTC AAG ATT   405
GAA AAG AAC GGC AAG GTC AGC GGG ACC AAG AAG GAA AAC TGT CCG TAC   453
AGT ATC CTA GAG ATA ACA TCA GTG GAA ATC GGA GTT GTT GCC GTC AAA   501
GCC ATT AAC AGC AAC TAT TAC TTA GCC ATG AAC AAG AAG GGG AAA CTC   549
TAT GCC TCA AAA GAA TTT AAC AAT GAC TGT AAA CTG AAA GAG AGG ATA   597
GAG GAA AAT GGA TAC AAC ACC TAT GCA TCT TTT AAC TGG CAG CAC AAC   645
GGC AGG CAA ATG TAT GTG GCA TTG AAT GGA AAA GGA GCT CCC AGG AGA   693
GGA CAA AAA ACA AGA AGG AAA AAC ACC TCC GCT CAC TTC CTC CCC ATG   741
GTG GTC CAC TCA TAGAAGA AGGCACCGTT GGTGGATGCA GTACAACCAA TGA CTCTTTG 800
CCAA

```

sequence number 4  
sequence length: 690 bp  
sequence form: nucleic acid  
topology: double-stranded chain  
sequence type: cDNA  
origin  
species name: human  
sequence

```

CTTCCAGTAT GTTCCTTCTG ATGAGACAAT TTCCAGTGCC GAGAGTTCCA GTACA ATG   58
TGG AAA TGG ATA CTG ACA CAT TGT GCC TCA GCC TTT CCC CAC CTG CCC   106
GGC TGC TGC TGC TGC TGC TTT TTG TTG CTG TTC TTG GTG TCT TCC GTC   154
CCT GTC ACC TGC CAA GCC CTT GGT CAG CAC ATG GTG TCA CCA GAG GCC   202
ACC AAC TCT TCT TCC TCC TCC TTC TCC TCT CCT TCC AGC GCG GGA AGG   250
CAT GTG CGG AGC TAC AAT CAC CTT CAA GGA GAT GTC CGC TGG AGA AAG   298
CTA TTC TCT TTC ACC AAG TAC TTT CTC AAG ATT GAG AAG AAC GGG AAG   346
GTC AGC GGG ACC AAG AAG GAG AAC TGC CCG TAC AGC ATC CTG GAG ATA   394
ACA TCA GTA GAA ATC GGA GTT GTT GCC GTC AAA GCC ATT AAC AGC AAC   442
TAT TAC TTA GCC ATG AAC AAG AAG GGG AAA CTC TAT GGC TCA AAA GAA   490
TTT AAC AAT GAC TGT AAG CTG AAG GAG AGG ATA GAG GAA AAT GGA TAC   538
AAT ACC TAT GCA TCA TTT AAC TGG CAG CAT AAT GGG AGG CAA ATG TAT   586
GTG GCA TTG AAT GGA AAA GGA GCT CCA AGG AGA GGA CAG AAA ACA CGA   634
AGG AAA AAC ACC TCT GCT CAC TTT CTT CCA ATG GTG GTA CAC TCA TAGAG 684
GAAGGC                                                                    690

```



sequence number 5  
sequence length: 22 bp  
sequence form: nucleic acid  
number of chain: single-stranded chain  
topology: linear chain  
sequence  
GATGCATAGG TATTGTATCC AT

sequence number 6  
sequence length: 21 bp  
sequence form: nucleic acid  
number of chain: single-stranded chain  
topology: linear chain  
sequence  
TCCATTTTCC TCTATCCTCT C

sequence number 7  
sequence length: 20 bp  
sequence form: nucleic acid  
number of chain: single-stranded chain  
topology: linear chain  
sequence  
AGAAGGGGAA ACTCTATGGC

sequence number 8  
sequence length: 21 bp  
sequence form: nucleic acid  
number of chain: single-stranded chain  
topology: linear chain  
sequence  
GACTGTAAAC TCAAAGAGAG G

sequence number 9

sequence length: 32 bp

sequence form: nucleic acid

number of chain: single-stranded chain

topology: linear chain

sequence

GCGAGCTCAA GCTTTTTTTT TTTTTTTTTT TT

sequence number 10

sequence length: 18 bp

sequence form: nucleic acid

number of chain: single-stranded chain

topology: linear chain

sequence

GCGAGCTCAA GCTTTTTT

sequence number 11

sequence length: 20 bp

sequence form: nucleic acid

number of chain: single-stranded chain

topology: linear chain

sequence

CTTCCAGTAT CATCCTTCTG

sequence number 12

sequence length: 20 bp

sequence form: nucleic acid

number of chain: single-stranded chain

topology: linear chain

sequence

GGCAAAGAGT CATTGTTGT

sequence number 13  
sequence length: 22 bp  
sequence form: nucleic acid  
number of chain: single-stranded chain  
topology: linear chain  
sequence  
GATGCATAGG TATTGTATCC AT

sequence number 14  
sequence length: 22 bp  
sequence form: nucleic acid  
number of chain: single-stranded chain  
topology: linear chain  
sequence  
GAAACTCTAT GGCTCAAAAG AA

sequence number 15  
sequence length: 20 bp  
sequence form: nucleic acid  
number of chain: single-stranded chain  
topology: linear chain  
sequence  
GTACACTCAT AGAGGAAGGC

sequence number 16  
sequence length: 34 bp  
sequence form: nucleic acid  
number of chain: single-stranded chain  
topology: linear chain  
sequence  
GGGAATTCCA TATGCTTGCT CAGGACATGG TGTC

sequence number 17

sequence length: 29 bp

sequence form: nucleic acid

number of chain: single-stranded chain

topology: linear chain

sequence

CGCGGATCCG CTATGCATGC AACGCGTTG

## Patent Claims

1. Recombinant DNA containing a nucleotide sequence encoding the polypeptide fibroblast growth factor whose amino acid sequence is represented by sequence number 1 or 2, or a nucleotide sequence complementary to the above nucleotide sequence.
2. The DNA described under Claim 1, containing the nucleotide sequence represented by sequence number 3 or 4 or a nucleotide sequence complementary to the above nucleotide sequence.
3. Expression vector containing the DNA described under Claim 1.
4. Transformant obtained by introducing the expression vector described under Claim 3 into a host.
5. The transformant described under Claim 4, in which the host is an animal cell or E. coli.
6. Method for producing recombinant fibroblast growth factor, characterized by use of the transformant described under Claim 4.
7. Recombinant fibroblast growth factor that is a polypeptide containing the amino acid sequence represented by sequence number 1 or 2 or the major portions.
8. Recombinant fibroblast growth factor that is a polypeptide containing the amino acid sequence represented by sequence number 1 or 2 or major portions of them, characterized by that it is produced by the transformant described under Claim 5 and that has cell growth-promoting activity.
9. Pharmaceutical preparation containing the recombinant fibroblast growth factor described under Claim 7 or 8 as active ingredient.

10. The pharmaceutical preparation described under Claim 9, that is a therapeutic agent for diseases of bone/cartilage or injuries of bone/cartilage.
11. The pharmaceutical preparation described under Claim 9, that is a wound healing-promoting agent.
12. Use of the recombinant fibroblast growth factor described under Claim 7 or 8 for the production of the therapeutic agent for diseases of bone/cartilage or injuries of bone/cartilage.
13. Use of the recombinant fibroblast growth factor described under Claim 7 or 8 for the production of a wound healing-promoting agent.
14. Method for treating diseases of bone/cartilage or injuries of bone/cartilage by administering an effective dose of the recombinant fibroblast growth factor described under Claim 7 or 8 in animals or humans.
15. Method for promoting wound healing by administering an effective dose of the recombinant fibroblast growth factor described under Claim 7 or 8 in animals or humans.

Figure 1

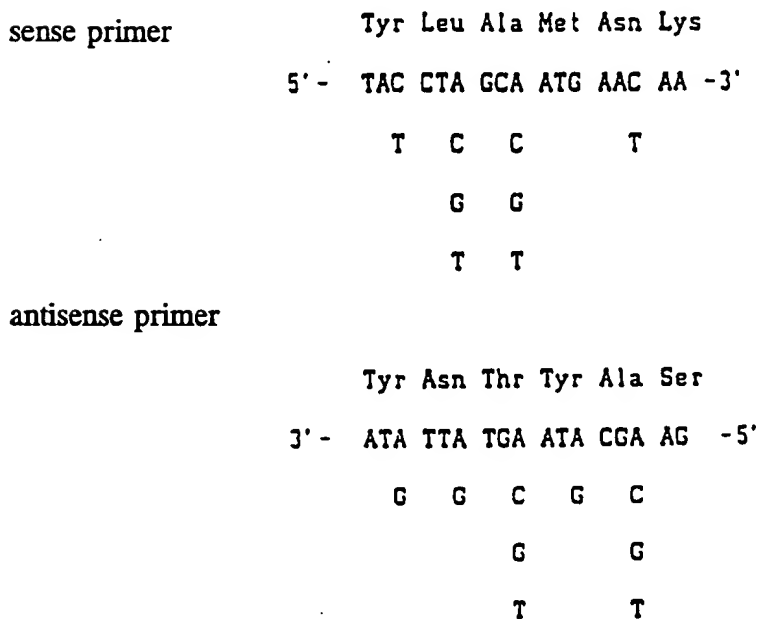


Figure 2

5' RACE method

A: 5'-CCT CTC TTT CAG TTT ACA GTC -3'  
A': 5'-GAT GCA TAG GTA TTG TAT CCA T-3'  
B: 5'-TCC ATT TTC CTC TAT CCT CTC -3'  
X: 5'-GCG AGC TCA AGC TTT TTT TTT TTT TTT TT-3'  
Y: 5'-GCG AGC TCA AGC TTT TTT -3'

3' RACE method

C: 5'-AGA AGG GGA AAC TCT ATG GC -3'  
D: 5'-GAC TGT AAA CTG AAA GAG AGG -3'  
D': 5'-GAA ACT CTA TGG CTC AAA AGA A-3'  
X: 5'-GCG AGC TCA AGC TTT TTT TTT TTT TTT TT-3'  
Y: 5'-GCG AGC TCA AGC TTT TTT -3'

for amplifying total sequence

E: 5'-CTT CCA GTA TGT TCC TTC TG-3'  
F: 5'-GGC AAA GAG TCA TTG GTT GT-3'  
F': 5'-GTA CAC TCA TAG AGG AAG GC-3'

Figure 3

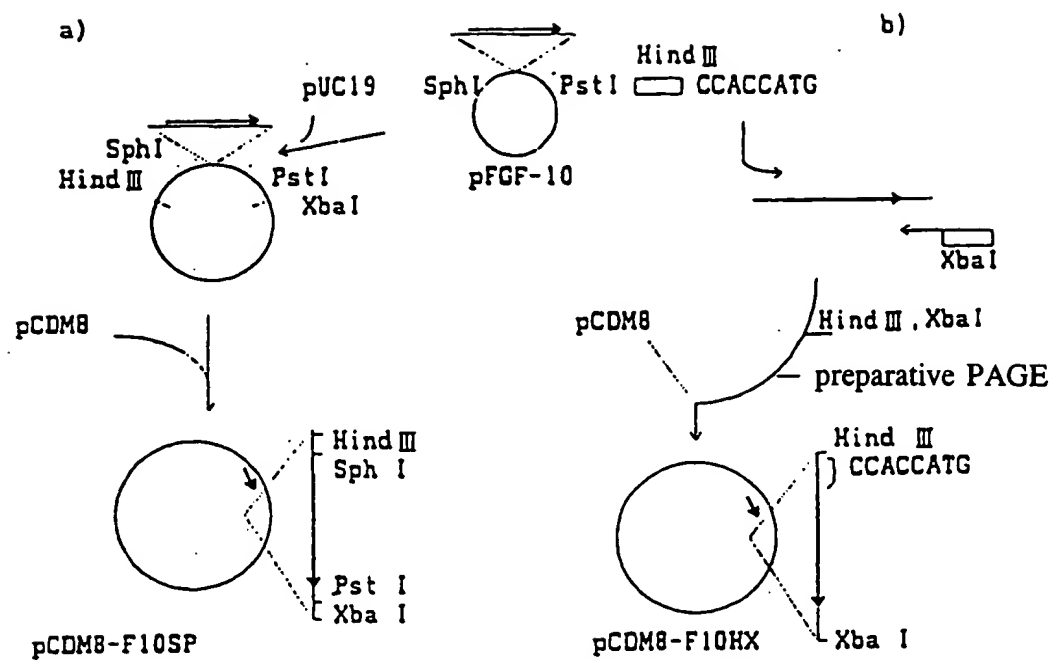




Figure 4

nucleotide sequences of the primers used for converting the region upstream of the deduced translation start site to Kozak consensus sequence

name	number of nucleotide	sequence (5'→3')
F10HS	35mer	TTTAAAGCTT CCACC ATGTGGAAGTGGATACTGAC
F10XR	27mer	AAAATCTAGA GTCATTGGTTGTACTGC

reaction conditions:

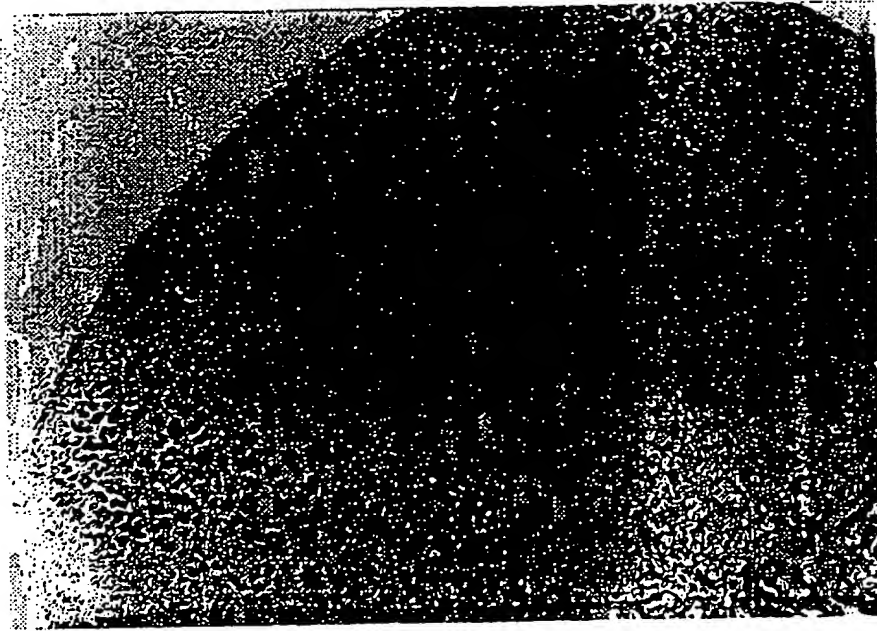
pFGF-10(0.5 $\mu$ g/ $\mu$ l)	2	$\mu$ l
10xPCR buffer	10	
10 $\mu$ M F10HS	2.5	
10 $\mu$ M F10XR	2.5	
dNTP mix(TaKaRa)	8	
dH2O	74.5	
AmpliTaq	0.5	/100 $\mu$ l

94℃	30 sec	
94℃	60 sec	] x 10回
56℃	60 sec	
72℃	60 sec	
72℃	60 sec	
	9 min	

mutation-introducing fragments

Figure 5

(A) Micrograph of ultrathin slice specimen of joint cartilage tissue



(B) Micrograph of ultrathin slice specimen of apophysis cartilage tissue

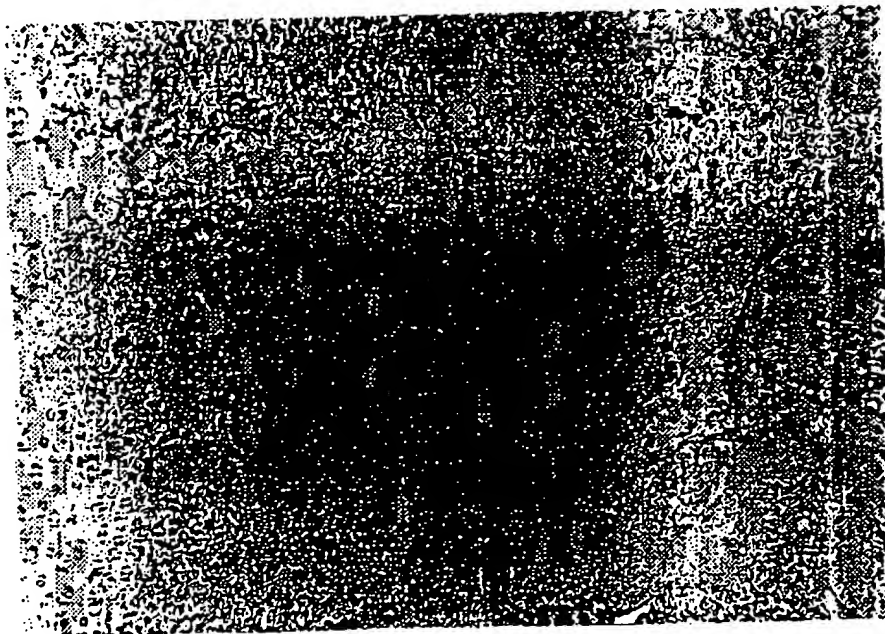
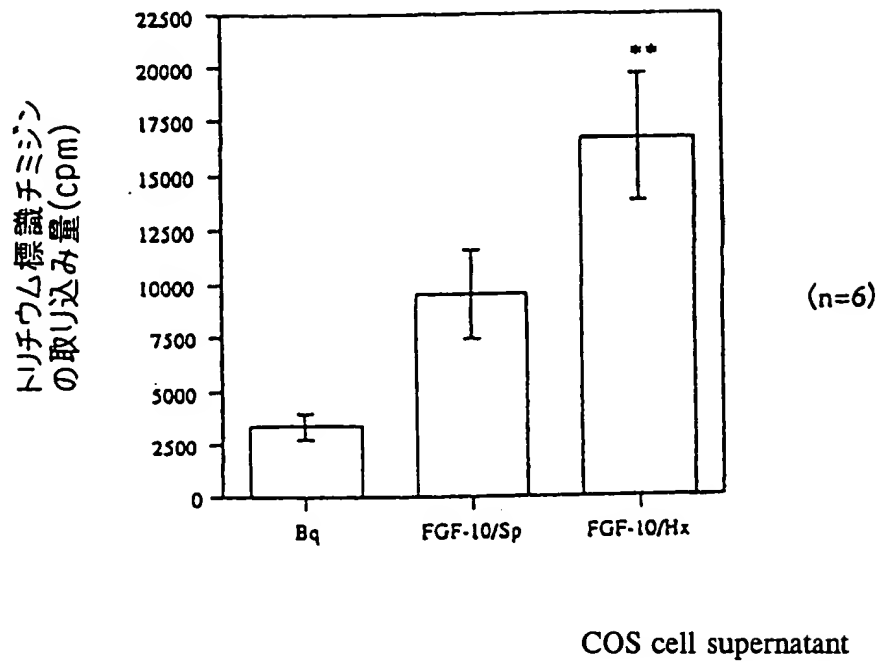


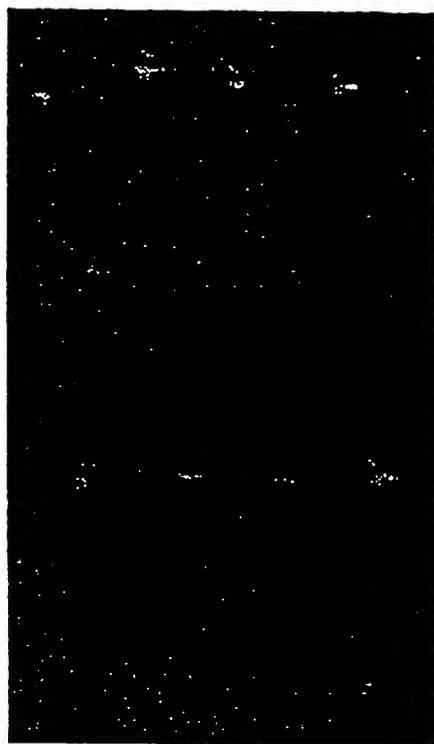
Figure 6  
Incorporation of tritium-labeled thymidine (cpm)



Changes of the incorporation of tritium-labeled thymidine in FRSK cells after the addition of COS cell supernatant

Values are mean  $\pm$  standard deviations.  $p < 0.01$  is considered significant (\*\*), compared to Bq.

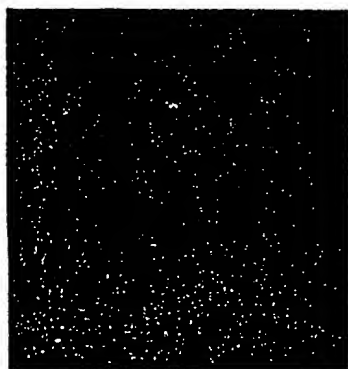
Figure 7



Processed image of soft X-ray picture of tibia in human FGF-10 administration group in Testing Example.

(Upper: 10.6  $\mu$ g of FGF-10; lower: 21.2  $\mu$ g of FGF-10)

Figure 8



Processed image of soft X-ray picture of tibia in control group in Testing Example.

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FIG.9

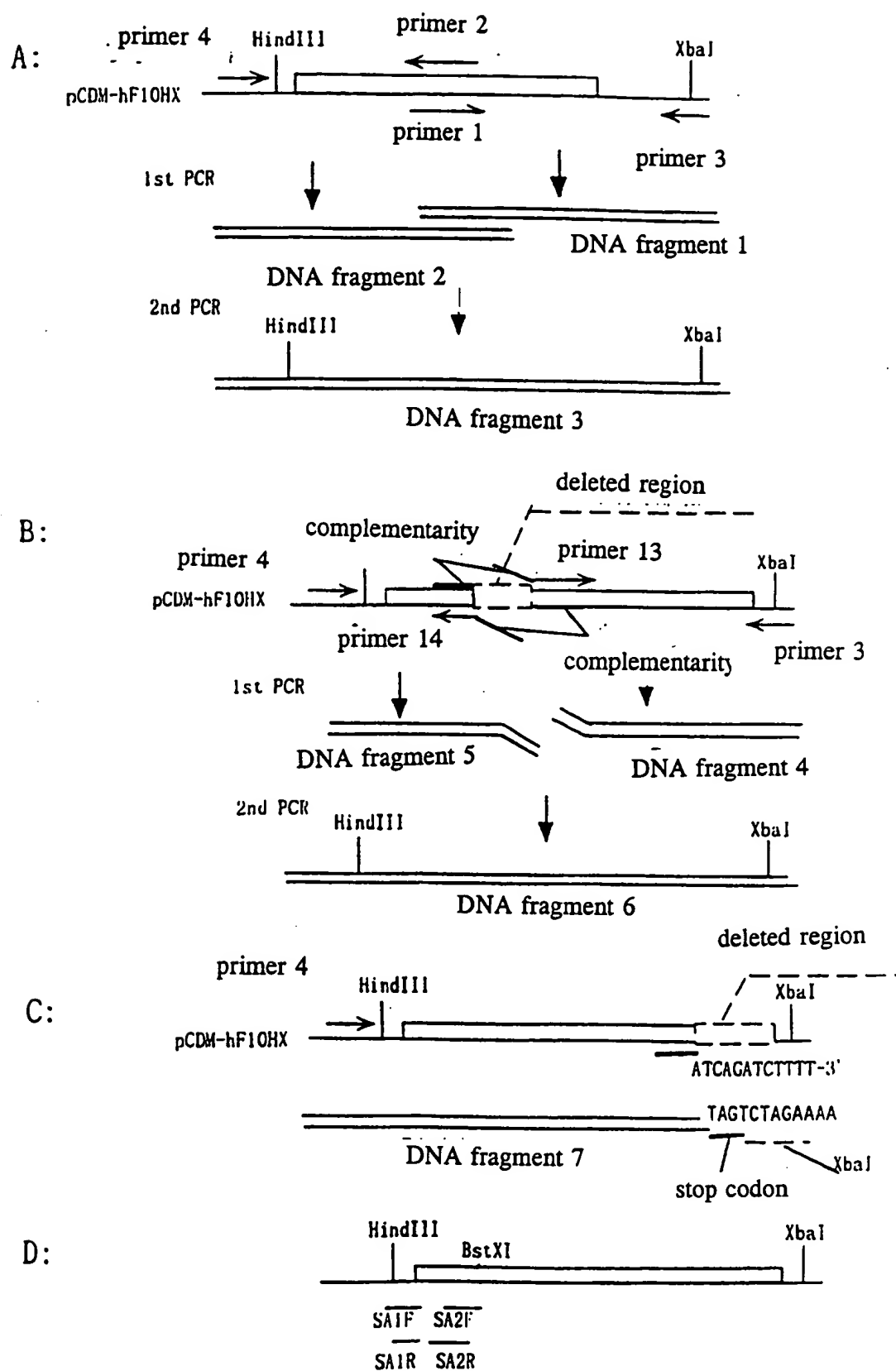
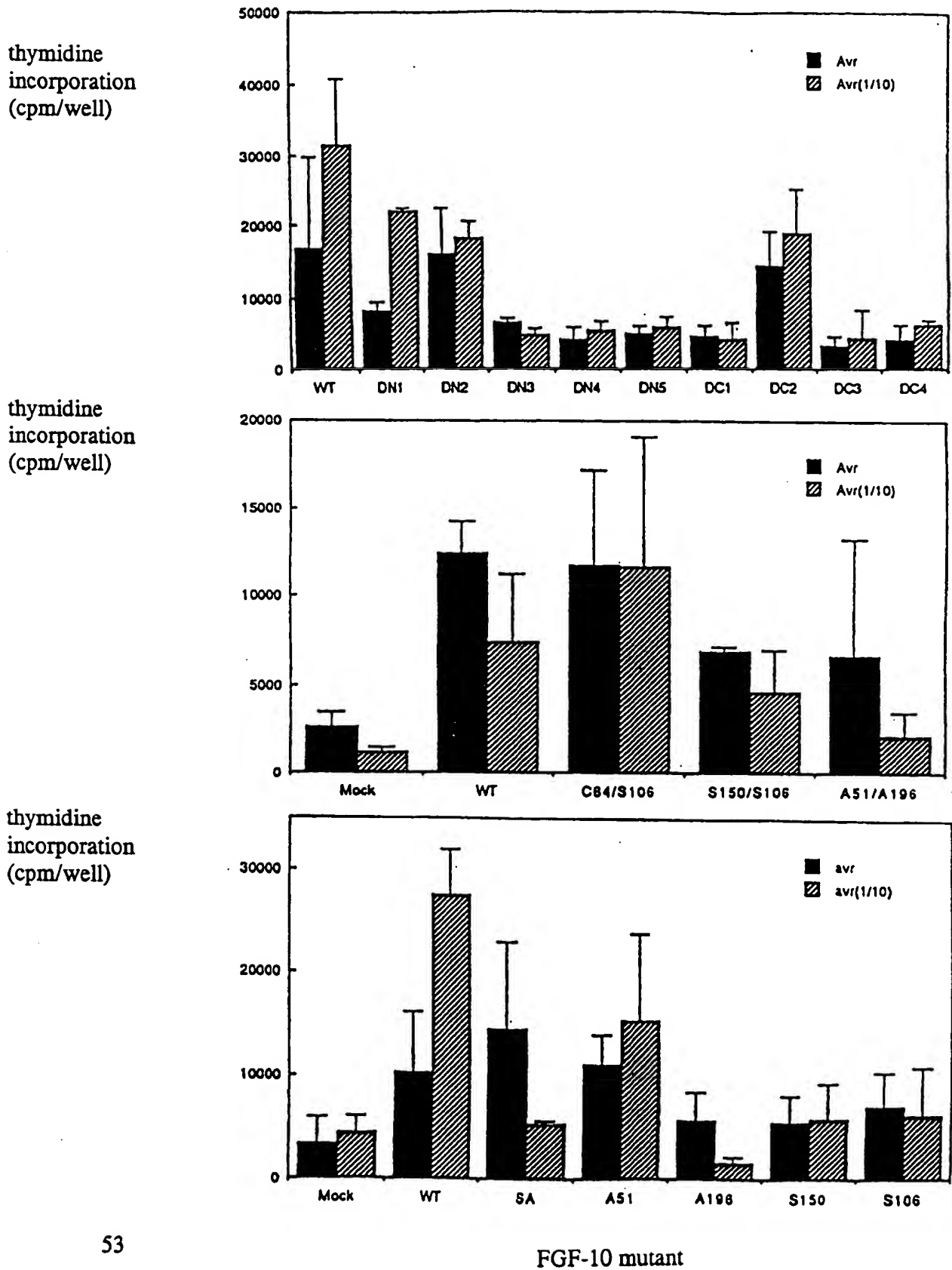


Figure 10

Mitogen activity of FGF-10 mutants for FRSK cells



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP96/03579

## A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl<sup>6</sup> C12N15/12, C12N5/10, C12N1/21, C12P21/02, C07K14/50, A61K37/36

According to International Patent Classification (IPC) r to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl<sup>6</sup> C12N15/12, C12N5/10, C12N1/21, C12P21/02, C07K14/50, A61K37/36

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS, WPI, Biosis previews

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO, A1, 96/25422 (Human Genome Sciences, Inc.), August 22, 1996 (22. 08. 96)	1 - 15
P,X	J. Biol. Chem., Vol. 271, No. 27, (1996. July), Yamasaki et al., "Structure and expression of the rat mRNA encoding a novel member of the fibroblast growth factor family" P. 15918-15921	1 - 15
A	Cell, Vol. 27, No. 9, (1995), P. 341-344	

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

February 18, 1997 (18. 02. 97)

Date of mailing of the international search report

February 25, 1997 (25. 02. 97)

Name and mailing address of the ISA/

Japanese Patent Office

Authorized officer

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